Structure–Activity Relationship Studies on the Mosquito Toxicity and Biting Deterrency of Callicarpenal Derivatives

by **Charles L. Cantrell***[a]), **Jerome A. Klun**[b]), **Julia Pridgeon**[c]), **James Becnel**[c]), **Solomon Green III**[a]), and **Frank R. Fronczek**[d])

[a]) USDA-ARS, Natural Products Utilization Research Unit, University, MS 38677, USA (phone: +1-662-915-5898; fax: +1-662-915-1035; e-mail: charles.cantrell@ars.usda.gov)

[b]) USDA-ARS, Beltsville Agricultural Research Center, Invasive Insects Biocontrol and Behavior Laboratory, Beltsville, MD 20705, USA

[c]) USDA-ARS, Mosquito and Fly Research Unit, Gainesville, FL 32608, USA

[d]) Department of Chemistry, Louisiana State University, Baton Rouge, Louisiana 70803, USA

Callicarpenal (=13,14,15,16-tetranorclerod-3-en-12-al=[(1*S*,2*R*,4a*R*,8a*R*)-1,2,3,4,4a,7,8,8a-octahydro-1,2,4a,5-tetramethylnaphthalen-1-yl]acetaldehyde; **1**) has previously demonstrated significant mosquito bite-deterring activity against *Aedes aegypti* and *Anopheles stephensi* in addition to repellent activity against host-seeking nymphs of the blacklegged tick, *Ixodes scapularis*. In the present study, structural modifications were performed on callicarpenal (**1**) in an effort to understand the functional groups necessary for maintaining and/or increasing its activity and to possibly lead to more effective insect control agents. All modifications in this study targeted the C(12) aldehyde or the C(3) alkene functionalities or combinations thereof. Mosquito biting deterrency appeared to be influenced most by C(3) alkene modification as evidenced by catalytic hydrogenation that resulted in a compound having significantly less effectiveness than **1** at a test amount of 25 nmol/cm^2. Oxidation and/or reduction of the C(12) aldehyde did not diminish mosquito biting deterrency, but, at the same time, none of the modifications were more effective than **1** in deterring mosquito biting. Toxicities of synthesized compounds towards *Ae. aegypti* ranged from an LD_{50} value of 2.36 to 40.11 μg per mosquito. Similarly, LD_{95} values ranged from a low of 5.59 to a high of 104.9 μg.

1. Introduction. – The *United States Centers for Disease Control and Prevention* (*CDC*) recommend the use of products containing active ingredients which have been registered with the *U.S. Environmental Protection Agency* (*EPA*) for use as repellents applied to skin and clothing. EPA Registration of repellent-active ingredients indicates that the materials have been reviewed, and approved for efficacy and human safety when applied according to the instructions on the label (http://www.cdc.gov/ncidod/dvbid/westnile/RepellentUpdates.htm). Of the active ingredients registered with the EPA, DEET (=*N*,*N*-diethyl-*m*-toluamide) and picaridin (=2-(2-hydroxyethyl)piperidine-1-carboxylic acid 1-methylpropyl ester) have arguably demonstrated higher degrees of efficacy in the peer-reviewed, scientific literature [1]. Also recognized by the CDC as effective insect repellents are those containing oil of lemon eucalyptus (primarily *p*-menthane-3,8-diol (PMD)), a plant-derived active ingredient. This recognition by the CDC is perhaps a testament to the changing perceptions in the United States, and the desire by consumers for effective and natural alternatives to conventional synthetic-based repellents.

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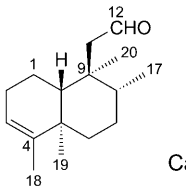
Similarly, there is an urgent need for the development of alternative insecticides to control important disease vectors such as *Aedes aegypti* (L.) (Diptera: Culicidae). *Ae. aegypti* transmits viral pathogens to humans, including yellow fever and dengue, both of which can cause severe human morbidity and mortality. One potential source of new insecticides is natural plant derivatives. Not only might certain natural plant products be a source of new pesticides, but also botanical chemical derivatives may be more environmentally friendly than synthetic chemicals.

In Mississippi, crushed leaves of American beautyberry, *Callicarpa americana* L. (Verbenaceae), were placed under the harnesses of draft animals as a traditional means to protect the animals from hematophagous insects [2][3]. Beautyberry leaves have been used as recently as the 1980s to repel arthropods (*Charles Bryson*, personal communication). *Cantrell et al.* [2] studied terpenoid compounds isolated from American and Japanese beautyberry, *C. japonica* THUNB., and discovered that at least two of these compounds, callicarpenal and intermedeol, had significant repellent activity against two species of mosquitoes and one tick species. The purpose of this study is to perform a structure–activity relationship study on both the mosquito biting deterrency and mosquito toxicity of synthetic analogs of callicarpenal. Callicarpenal is currently being investigated by the *United States Department of Agriculture*'s *Agricultural Research Service* (*USDA-ARS*) as a possible alternative to commercially available natural and synthetic-based insect repellents [4][5]. Discussed below are synthetic modifications performed to callicarpenal including oxidations and reductions of the C(12) aldehyde to its corresponding acid or alcohol, complete reduction of the C(3) alkene, epoxidation of the C(3) alkene, and various methyl ester and acetate products. Combinations of the above modifications will also be discussed, as well as their effects on both *Ae. aegypti* biting-deterrency and toxicity.



Callicarpenal (**1**)

2. Results and Discussion. – Bulk purification of callicarpenal (**1**) was performed prior to beginning the structure–activity analysis. A three-step approach was utilized consisting of steam distillation of *Callicarpa americana* leaves using a *Clevenger* apparatus, followed by two normal-phase chromatographic purification steps which resulted in >3 g of **1** (*ca.* 94–98% chromatographic purity) for the study. At various stages in the purification process, the authors attempted to use crystallization to expedite the process; however, success was not achieved until all chromatographic purifications were complete. Crystallization was only achieved when 1 ml of Et_2O was allowed to evaporate slowly at $-5°$ when combined with >800 mg of pure (*ca.* 94–98% chromatographic purity) **1**. *Fig. 1* shows the X-ray-determined structure of **1** with 50% ellipsoids reported here for the first time. Structural confirmation using MS, and ^{1}H- and ^{13}C-NMR data was performed as described in [2].

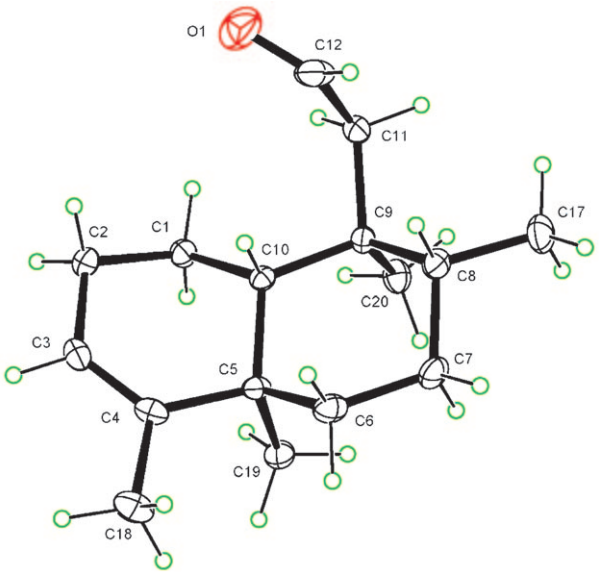


Fig. 1. *X-Ray structure of callicarpenal* (**1**)*, with 50% ellipsoids*

Reduction of **1** using H_2 and 5% Pd/C cleanly produced the desired product, 13,14,15,16-tetranorclerodan-12-al (**2**; *Scheme*). Positive-ion high-resolution (HR) MS analysis gave a parent molecular ion at *m/z* 259.2008 corresponding to $[M+Na]^+$ and suggesting a molecular formula of $C_{16}H_{28}O$. ^{1}H-NMR Analysis indicated the olefinic H-atom at C(3), and the olefinic Me *singlet* present in the spectrum of **1** were no longer present in **2**. Instead, a new Me *doublet* was now present in the spectrum of **2** at δ 0.71 (3 H). ^{13}C- and DEPT-NMR analysis confirmed the presence of the aliphatic Me group, and the addition of one CH_2 and one CH aliphatic C-atoms (C(3) and C(4)) relative to **1**. Compound **2** was further reduced using $NaBH_4$ providing a compound with a molecular-ion peak at *m/z* 238 (M^+). Both positive- and negative-ion HR-MS analysis was unsuccessful; however, X-ray crystallographic analysis was successful and was not only used to confirm the molecular formula and structure, but also used to unequivocally assign the orientation of the Me group at C(4) as α (*Fig. 2*) in both **3** and, by inference, also in **2**. ^{1}H- and ^{13}C-NMR analysis were both in agreement with the structure as that of 13,14,15,16-tetranorclerodan-12-ol (**3**). Compound **2** is reported here for the first time; however, its enantiomer had been reported previously reported [6]. Compound **3** is reported here for the first time.

A $NaBH_4$ reduction was also performed (*Scheme*) directly on **1**, providing a compound giving a positive-ion MS parent molecular ion peak at *m/z* 237.2059 ($[M+H]^+$) and suggesting a molecular formula of $C_{16}H_{28}O$. The ^{1}H-NMR analysis indicated the aldehyde signal present in the spectrum of **1** was not present in that of this product as expected. ^{13}C-NMR and DEPT analysis indicated the absence of the aldehyde C=O group and the presence of a CH_2 signal at δ 58.9 corresponding to C(12) and helping to confirm the structure as that of 13,14,15,16-tetranorclerod-3-en-12-ol (**4**). Compound **4** had been reported previously as a synthetic intermediate during the total synthesis of

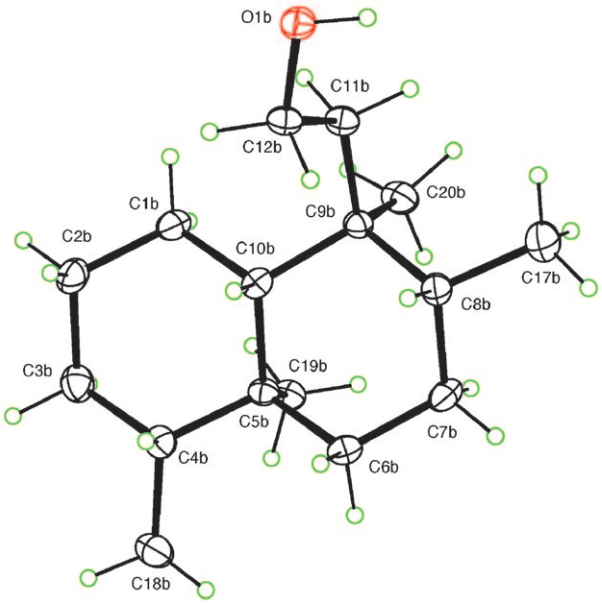
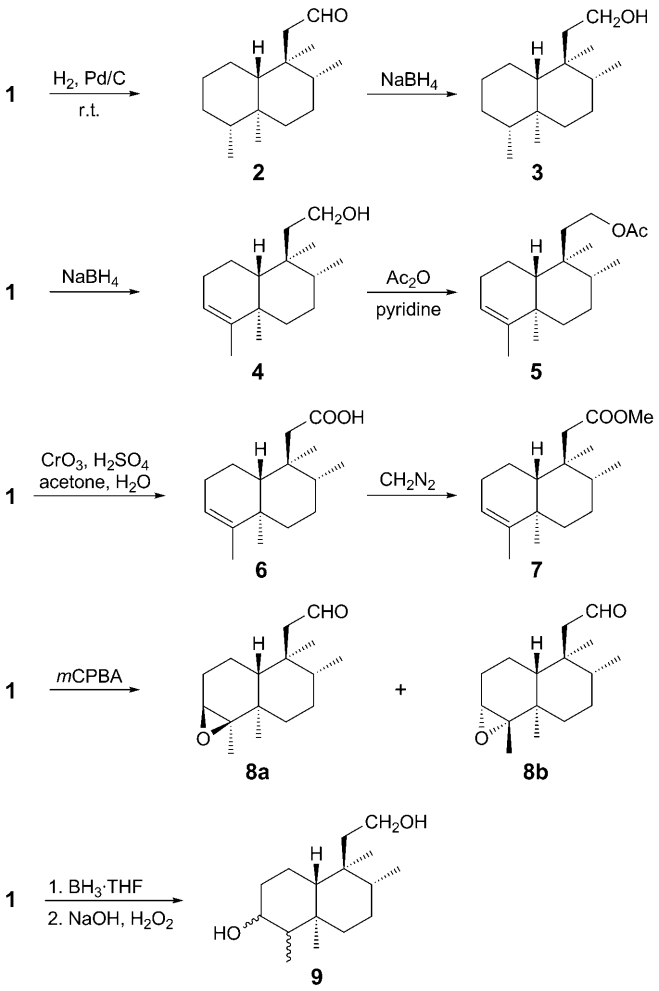


Fig. 2. *X-Ray structure of one of the two independent molecules of* **3***, with 50% ellipsoids*

the (14*Z*)-16-hydroxycleroda-3,13(14)-dien-15,16-olide. ^{1}H-NMR Data were in agreement with those reported previously [7], while ^{13}C-NMR data are reported here for the first time. Acetylation of **4** with Ac_2O in pyridine provided a compound giving a positive-ion MS parent molecular ion of *m*/*z* 301.2121 corresponding to $[M+Na]^+$ and suggesting the desired molecular formula of $C_{18}H_{30}O_2$ for **5**. ^{1}H-NMR Analysis of **5** indicated a 3-H *singlet* at δ 1.98 and corresponding to the acetate Me group. ^{13}C-NMR Analysis further confirmed the presence of this Me and an additional C=O group (δ 171.2) confirming the addition of an acetate. COSY, HMQC, and HMBC analyses confirmed the attachment of the acetate to C(12) and established the structure as **5**. Compound **5** is reported here for the first time.

Jones oxidation of **1** (*Scheme*) provided a compound giving a strong $[M-H]^-$ molecular-ion peak at *m*/*z* 249.1978 in negative-ion HR-MS analysis, suggesting the desired product had been produced and had a molecular formula of $C_{16}H_{26}O_2$. ^{1}H- and ^{13}C-NMR analyses indicated that the aldehyde signals for C(12) in **1** were no longer present, instead a signal of carboxylic acid C-atom was present at δ 178.8 corresponding to C(12). DEPT, COSY, HMQC, and HMBC analyses confirmed the structure as that of 13,14,15,16-tetranorclerod-3-en-12-oic acid (**6**). ^{1}H- and ^{13}C-NMR data for the enantiomer of compound **6**, a synthetic intermediate produced during the total synthesis of avarol and avarone [8][9], were in complete agreement with those reported for its enantiomer. Methylation of **6** using CH_2N_2 provided a compound giving a parent molecular-ion peak at *m*/*z* 265.2166 in positive-ion HR-MS analysis and corresponding to $[M+H]^+$. This information, together with DEPT and ^{13}C-NMR analysis, suggested a molecular formula of $C_{17}H_{28}O_2$. ^{1}H-NMR Analysis revealed the

Scheme. *Modifications to Callicarpenal* (**1**)



presence of the Me group of the newly introduced ester (δ 3.58 (3 H)). The above data together with 2D-NMR data (HSQC, HMBC, and COSY) established the structure as that of **7**.

Peracid oxidation of **1** using *meta*-chloroperbenzoic acid (*m*CPBA) provided two products, as expected. When analyzed by HR-ESI-MS (positive-ion), the two products gave molecular-ion peaks at *m*/*z* 251.2101 (compound **8a**) and 251.2019 (compound **8b**) both corresponding to $[M+H]^+$ and suggesting a molecular formula of $C_{16}H_{26}O_2$. ^{1}H-, ^{13}C-, and DEPT-NMR analyses revealed that signals corresponding to the C(3)=C(4) bond were no longer present in either compound. Instead they had been replaced by those of an oxygenated CH group (C(3)) and an oxygenated C-atom (C(4)). The above data together with COSY, HMQC, and HMBC NMR data indicated that the desired

products **8a** and **8b** had been produced. Orientation of the 3,4-epoxide was deduced from structural observations of minimized **1** where it is predicted that epoxidation would occur predominantly from the top providing **8a** as the major product, which agrees with the results. Similarly, the results from the catalytic hydrogenation of **1** above indicated that attack from the top was strongly preferred providing exclusively compound **2**, as confirmed by X-ray crystallography of **3**. This is the first report on compounds **8a** and **8b**.

Lastly, hydroboration of **1** did not provide the intended product; however, the compound was included in this study. When analyzed by HR-ESI-MS (positive-ion), the major product gave a parent molecular ion adduct at *m/z* 277.2129 corresponding to $[M+\mathrm{Na}]^+$ and indicated a molecular formula of $C_{16}H_{30}O_2$. ^{1}H- and ^{13}C-NMR analyses indicated that the aldehyde functional group had been reduced to the corresponding alcohol, while the C(3) olefin had been substituted correctly. DEPT, COSY, HMQC, and HMBC analyses confirmed the structure as that of **9**. Compound **9** had not been reported previously.

The above derivatives of callicarpenal (**1**) were evaluated for toxicity towards *Ae. aegypti* (*Table 1*). Both LD_{50} and LD_{95} values were determined for all compounds. Toxicities of synthesized compounds ranged from an LD_{50} of 2.36 μg for **8a** to 40.11 μg for **8b**. Similarly, LD_{95} values ranged from a low of 5.59 μg for **8a** to a high of 104.92 μg for **8b**. Hydrogenation of **1** to **2** appeared to have little effect on toxicity, as LD_{50} values were not significantly different; however, LD_{95} values indicated that **2** was slightly more active than **1**. Further reduction of **2** to its corresponding alcohol **3** provided a much less active compound than either **1** or **2**. Furthermore, simply reducing callicarpenal (**1**) to the corresponding alcohol **4** provided a compound not significantly different than **1**. Acetylation of **4**, *Jones* oxidation of **1**, and methylation of **6** produced the desired products **5**, **6**, and **7**. LD_{50} Data indicated that all three of these compounds were significantly less active than callicarpenal (**1**), while LD_{95} data indicated no significant difference between these compounds and **1**. Lastly, epoxidation of **1** with *m*CPBA produced the desired products **8a** and **8b**. Compound **8a** with an LD_{50} value of 2.36 μg was significantly more active than **1**, while compound **8b** was much less active with an LD_{50} value of 40.11 μg.

Table 1. *Toxicities of Callicarpenal* (**1**) *and Analogs against Female* Aedes aegypti

Compound	LD_{50} (95% C.I.)[a])	LD_{95} (95% C.I.)[a])	Slope (SE)	χ^2
1	5.13 (1.83–7.26)	23.91 (15.98–87.90)	2.46 (0.73)	0.32
2	5.87 (4.98–6.88)	12.49 (9.98–18.59)	5.01 (0.83)	0.56
3	19.62 (12.31–26.57)	117.19 (63.76–802.35)	2.12 (1.13)	0.59
4	4.05 (0.51–6.51)	26.36 (16.34–212.63)	2.02 (0.70)	0.28
5	9.88 (8.12–11.71)	23.76 (18.64–36.58)	4.32 (0.73)	1.24
6	7.63 (6.28–9.60)	21.93 (15.36–45.43)	3.59 (0.68)	0.46
7	8.95 (7.02–10.83)	24.72 (18.47–44.86)	3.73 (0.74)	0.42
8a	2.36 (2.00–2.80)	5.59 (4.39–8.30)	4.40 (0.64)	1.00
8b	40.11 (32.49–48.15)	104.92 (79.51–178.83)	3.94 (0.72)	0.10
Piperine	8.13 (6.10–12.99)	58.74 (28.13–303.44)	1.92 (0.39)	0.54

[a]) LD_{50} and LD_{95} values are in units of μg of chemical per mosquito.

During the course of three separate experiments, the above compounds were also evaluated for biting deterrency against *A. aegypti* (*Table 2*) to determine their effectiveness relative to callicarpenal (**1**). All experiments included a solvent control, the positive control callicarpenal (**1**), and were performed at a single concentration of 25 nmol/cm^2. *Exper. 1* compared the effectiveness of **1** relative to that of compounds **2**, **8a**, and **8b**. Interestingly, hydrogenation of the C(3)=C(4) bond resulted in a much less active compound **2**, while epoxidation produced two isomers, **8a** and **8b**, with activity equal to that of **1**. *Exper. 2* compared the result of modification of the C(12) aldehyde in **1** to the corresponding alcohol, **4**, and acid, **6**. The data indicate that both **4** and **6** were as effective as callicarpenal (**1**). In *Exper. 3*, compounds **3**, **5**, and **9** were all less active than **1**; however, the activity was still significant above solvent control. Compound **7** was the only compound which demonstrated activity significant to **1**.

Table 2. *Mosquito-Deterrent Effects Using Callicarpenal* (**1**) *and Its Analogs against* Aedes aegypti[a])

Exper.	Treatment	Mean proportion not biting (SE)
1 (n=120)	Solvent control	0.31 (0.042)
	1	0.73 (0.040)[b])
	2	0.38 (0.044)[c])
	8a	0.70 (0.042)[b])[d])
	8b	0.68 (0.043)[b])[d])
2 (n=60)	Solvent control	0.40 (0.063)
	1	0.70 (0.059)[b])
	4	0.68 (0.060)[b])[d])
	6	0.60 (0.063)[b])[d])
3 (n=80)	Solvent control	0.31 (0.046)
	1	0.89 (0.031)[b])
	3	0.54 (0.050)[b])
	5	0.69 (0.046)[b])
	7	0.81 (0.039)[b])[d])
	9	0.55 (0.050)[b])

[a]) SE = Standard error. n = Number of mosquitoes tested against each treatment. [b]) Significantly different from solvent control (EtOH). [c]) Not different from control. [d]) Not different from positive control (callicarpenal (**1**)).

Experimental Part

General. Column chromatography (CC): *Biotage, Inc. Horizon™ Pump* (Charlottesville, Virginia) equipped with a *Horizon™ Flash Collector* and fixed-wavelength (254 nm) detector. HPLC: *Agilent 1100* system equipped with a quaternary pump, autosampler, diode-array detector, and vacuum degasser. Semi-prep. HPLC: *Waters Delta-Prep* system (Milford, MA) equipped with a diode-array detector and a binary pump. ^{1}H- and ^{13}C-NMR Spectra: in $CDCl_3$ or CD_3OD on a *Varian ANOVA* 400-MHz spectrometer (Palo Alto, CA); δ in ppm rel. to Me_4Si, J in Hz; all ^{13}C multiplicities were deduced from 90° and 135° DEPT experiments. EI-MS (70 eV): *Varian CP-3800* GC coupled to a *Varian Saturn-2000* MS/MS; in m/z (rel. %). HR-MS: *Agilent 1100* HPLC coupled to a *JEOL AccuTOF* (*JMS-T100LC*; Peabody, MA); in m/z.

X-Ray Crystal-Structure Analysis. The crystal structures of compounds **1** and **3** were determined using data collected at $T=90$ K with MoK_α radiation on a *Nonius KappaCCD* diffractometer. *Crystal data:* **1**: $C_{16}H_{26}O$, monoclinic space group $P2_1$, $a=7.5730(15)$, $b=9.924(2)$, $c=9.882(2)$ Å, $\beta=112.415(11)^\circ$, $V=686.6(2)$ Å^3, $Z=2$, $R=0.038$ (1654 data with $F^2>2\sigma$), $R_w=0.087$ (all F^2) for 1869 unique data having $\theta<28.7^\circ$ and 159 refined parameters; **3**: $C_{16}H_{30}O$, orthorhombic space group $P2_12_12_1$, $a=7.3989(10)$, $b=17.060(2)$, $c=23.455(3)$ Å, $V=2960.6(7)$ Å^3, $Z=8$, $R=0.044$ (3119 data with $F^2>2\sigma$), $R_w=0.096$ (all F^2) for 3990 unique data having $\theta<27.9^\circ$ and 322 refined parameters. Crystallographic data have been deposited with the *Cambridge Crystallographic Data Centre* and allocated the deposition numbers CCDC-724266 and 724267. Copies of the data can be obtained, free of charge, on application to the Director, CCDC, 12 Union Road, Cambridge CB21EZ, UK (fax: +44-(0)1223-336033 or e-mail: deposit@ccdc.cam.ac.uk).

High-Resolution (HR) LC/MS Analysis. All isolated compounds were prepared in MeOH and injected directly into a 0.3-ml/min stream of either MeOH or 80% MeOH/20% deionized H_2O. Twenty µl of sample (*ca.* 0.1 mg/ml) was injected manually at 0.5 min while mass drift compensation standards (L-tryptophan (negative ion), PEG (positive ion)) were injected at 1.5 min over the course of a 2-min run.

GC/MS Analysis. GC was equipped with a *DB-5* column (30 m × 0.25 mm fused silica cap. column, film thickness of 0.25 µm) operated using the following conditions: injector temp., 240°; column temp., 60–240° at 3°/min then held at 240° for 5 min; carrier gas, He; injection volume, 1 µl (splitless). MS Mass range from 40 to 650 *m*/*z*, filament delay of 3 min, target TIC of 20,000, a prescan ionization time of 100 µs, an ion trap temp. of 150°, manifold temp. of 60°, and a transfer line temp. of 170°.

Essential-Oil Preparation. Leaves of *C. americana* were collected in September and October of 2006 from multiple plants (*ca.* 4 m tall × 5 m wide) growing in Lafayette County, Mississippi, near latitude 34°20′25″N and longitude 89°40′17″W. A voucher specimen was previously deposited with the Pullen Herbarium in Oxford, Mississippi, and assigned voucher number MISS #71,495 [2]. Fresh-cut leaves of *C. americana* were immediately frozen in sealed plastic bags upon collection until needed. A *Clevenger*-type volatile oil distilling apparatus (*Wilmad Labglass*, Buena, NJ) was attached to a 12-l round-bottomed flask containing *C. americana* leaves (900.5 g fresh weight) in 6 l of deionized H_2O. Upon heating to boiling, the distillate was continuously extracted during a 168-h distillation with 6 ml of pentane providing 2.06 g of crude essential oils. This process was repeated as needed to obtain additional oil for fractionation and crystallization.

Purification and Crystallization of Callicarpenal (= *[(1S,2R,4aR,8aR)-1,2,3,4,4a,7,8,8a-Octahydro-1,2,4a,5-tetramethylnaphthalen-1-yl]acetaldehyde*; **1**). A portion (1.502 g) of the *C. americana* essential oil was subjected to silica-gel (40 × 150 mm, 60 Å, 40–63 µm) CC. A hexane/AcOEt linear gradient consisting of the following steps was used: 100 : 0 to 90 : 10, 1200 ml; 90 : 10 to 80 : 20, 600 ml; 80 : 20 to 50 : 50, 360 ml; 50 : 50 to 0 : 100, 1008 ml. A total of 132 24-ml test tubes were collected and combined into six fractions (*Fr. A*, 985 mg; *Fr. B*, 145 mg (crude callicarpenal); *Fr. C*, 24 mg; *Fr. D*, 30 mg; *Fr. E*, 68 mg; *Fr. F*, 176 mg) based on TLC similarity. This process was repeated providing additional crude callicarpenal. *Fr. B* (250 mg) was further purified using a hexane/CH_2Cl_2 linear gradient consisting of the following steps: 75 : 25 to 25 : 75, 1728 ml; 75 : 25 to 0 : 100, 1152 ml. A total of 120, 24-ml test tubes were collected and combined into three fractions (*Fr. A*, 24 mg; *Fr. B*, 110 mg (pure callicarpenal); and *Fr. C*, 107 mg) based on TLC similarity. This process was repeated as needed to obtain sufficient quantities of callicarpenal for bioassays. Crystallization was accomplished by dissolving 1.2 g of **1** in a few drops of Et_2O and allowing Et_2O to slowly evaporate at −4°.

X-Ray Crystal Structure of Callicarpenal (**1**). The crystal structure of **1** is illustrated in *Fig. 1*. The ring containing the C(3)=C(4) unsaturation has a twist conformation, in which C(2), C(3), C(4), and C(5) are coplanar to within an average deviation of 0.006(2) Å. The other two atoms lie on opposite sides of this plane, C(1) by 0.319(2) Å and C(10) by 0.486(2) Å. The saturated ring has a chair conformation, with endocyclic torsion-angle magnitudes in the range 51.7(2)–57.0(2)°. The C=O bond length is 1.194(3) Å, and the conformation of the aldehyde-containing substituent is described by torsion angles C(20)–C(9)–C(11)–C(12) 174.4(2) and C(9)–C(11)–C(12)–O(1) −115.2(2)°.

Catalytic Hydrogenation of **1** *to [(1S,2R,4aS,5R,8aS)-Decahydro-1,2,4a,5-tetramethylnaphthalen-1-yl]acetaldehyde* (**2**). Compound **1** (193 mg) was dissolved in 5 ml of MeOH in a 25-ml round-bottomed flask. Approximately 20 mg of 5% Pd/C was added to the mixture and charged with H_2 with stirring for

3.0 h. The mixture was filtered through a bed of *Celite 545* and washed with CH_2Cl_2. The reaction products were purified using normal-phase chromatography (*Biotage 25+M* column, 25–63 μm, 60 Å, 25 × 150 mm) running at 25 ml/min using a hexane/AcOEt step gradient from 100:0 to 50:50 over 1401 ml, and finishing with 50:50 to 0:100 over 600 ml. 24-ml Fractions were collected and recombined based on TLC similarities into two distinct compounds. Drying provided 23 mg of pure **2**. ^{1}H-NMR ($CDCl_3$): 9.80 (*t*, *J* = 4.0, 1 H); 2.39 (*dd*, *J* = 3.6, 14.8, 1 H); 2.25 (*dd*, *J* = 3.6, 14.8, 1 H); 0.90 (*d*, *J* = 6.8, 3 H); 0.77 (*s*, 3 H); 0.76 (*s*, 3 H); 0.71 (*d*, *J* = 6.4, 3 H). ^{13}C-NMR ($CDCl_3$): 203.9 (*s*); 52.8 (*d*); 51.7 (*t*); 45.8 (*d*); 41.76 (*s*); 39.3 (*d*); 38.9 (*t*); 37.5 (*s*); 30.6 (*t*); 27.2 (*t*); 27.0 (*s*); 22.1 (*t*); 17.2 (*q*); 16.4 (*q*); 15.0 (*q*); 13.1 (*q*). EI-MS: 236 (0, M^+), 193 (26), 192 (34), 177 (100), 163 (9), 18 (11). HR-ESI-MS (pos.): 259.2008 ($[M+Na]^+$, $C_{16}H_{28}NaO^+$; calc. 259.2038).

$NaBH_4$ Reduction of **2** *to 2-[(1*S*,2*R*,4a*S*,5*R*,8a*S*)-Decahydro-1,2,4a,5-tetramethylnaphthalen-1-yl]ethanol* (**3**). Compound **2** (48.0 mg) was dissolved in 7 ml of dry MeOH and transferred to a dry round-bottomed flask under stirring. To this soln. in an ice bath was slowly added 380 mg of $NaBH_4$. The reaction was monitored by TLC every 15 min until compound **2** was completely used up. Reaction was complete in 3.0 h at which time *ca.* 10 ml of deionized H_2O was used to quench the reaction. This mixture was extracted three times with 15 ml of $CHCl_3$. After removal of organics *in vacuo*, the residue was separated by normal-phase CC (silica gel, 40–63 μm, 60 Å, 40 × 150 mm) on a *Biotage 40+M* column running at 40 ml/min using a hexane/AcOEt step gradient beginning with 100:0 to 50:50 over 1800 ml, followed by 50:50 to 0:100 over 600 ml. 24-ml Fractions were collected and recombined based on TLC similarities into four distinct fractions (*A* to *D*). *Fr. D* provided 35.5 mg of pure **3**. ^{1}H-NMR ($CDCl_3$): 3.62–3.48 (*m*, 2 H); 0.80 (*d*, *J* = 6.4, 3 H); 0.72 (*s*, 3 H); 0.68 (*d*, *J* = 6.4, 3 H); 0.66 (*s*, 3 H). ^{13}C-NMR ($CDCl_3$): 58.8 (*t*); 51.1 (*d*); 46.2 (*d*); 41.0 (*t*); 39.4 (*t*); 38.9 (*s*); 37.8 (*d*); 37.4 (*s*); 30.9 (*t*); 27.6 (*t*); 27.5 (*t*); 21.9 (*t*); 18.2 (*q*); 16.5 (*t*); 15.3 (*q*); 13.4 (*q*). EI-MS: 238 (1, M^+), 193 (100), 192 (27), 137 (49), 123 (58), 109 (56), 81 (47).

X-Ray Crystal Structure of **3**. The structure of one of the two independent molecules in the asymmetric unit of **3** is shown in *Fig. 2*. The two molecules, A and B, have nearly identical conformations, with *trans*-fused rings in the chair conformation. Endocyclic torsion-angle magnitudes fall within the range 50.1(2)–59.8(2)°, and their mean difference between the two molecules is only 1.2°. Unlike in **1**, the O-carrying substituent at C(9) is fully extended. The largest conformational difference between the two independent molecules is the torsion angle about C(11)–C(12), which is 163.5(2)° in one molecule and 171.9(2)° in the other. The difference is apparently a result of H-bonding. The OH groups form intermolecular H-bonds with O···O distances 2.694(2) and 2.698(2) Å. These contacts are nearly linear, with angles about H 169(2) and 177(3)°, and form chains of alternating A and B molecules in the [1 0 0] crystal direction. The fact that a single OH group must serve as both H-bond donor and acceptor appears to be the cause of the two independent molecules in the crystal. *Brock* and *Duncan* have studied packing of monoalcohols, and found that occurrence of more than one molecule in the asymmetric unit is common [10].

$NaBH_4$ Reduction of **1** *to 2-[(1*S*,2*R*,4a*R*,8a*R*)-1,2,3,4,4a,7,8,8a-Octahydro-1,2,4a,5-tetramethylnaphthalen-1-yl]ethanol* (**4**). Compound **1** (237.7 mg) was dissolved in 60 ml of dried MeOH and transferred to a dry 100-ml round-bottomed flask with a magnetic stirrer. To this soln., 1.4 g of $NaBH_4$ was slowly added in an ice bath. TLC (hexane/Et_2O 8:2, *Godin* rgt) was performed every 15 min, until starting material was completely consumed. The reaction was complete in 85 min, at which time *ca.* 25 ml of deionized H_2O was used to quench the reaction. This mixture was extracted twice with 30 ml of $CHCl_3$. After removal of solvent *in vacuo*, the residue (237.5 mg) was separated by silica-gel CC on a *Biotage 40+M* column (40–63 μm, 60 Å, 40 × 150 mm) running at 40 ml/min using a hexane/AcOEt step gradient beginning with 100:0 to 50:50 over 1800 ml, followed by 50:50 to 0:100 over 600 ml. 24-ml Fractions were collected and recombined based on TLC similarities into four distinct fractions (*A* to *D*). *Fr. D* provided 84.4 mg of pure **4**. ^{1}H-NMR ($CDCl_3$): 5.22 (br. *s*, 1 H); 3.70–3.60 (*m*, 2 H); 2.15–1.95 (*m*, 2 H); 1.61 (br. *s*, 3 H); 1.03 (*s*, 3 H); 0.90 (*d*, *J* = 6, 3 H); 0.78 (*s*, 3 H). ^{13}C-NMR ($CDCl_3$): 144.5 (*s*); 120.7 (*d*); 58.9 (*t*); 47.8 (*d*); 41.2 (*t*); 39.0 (*s*); 38.5 (*s*); 37.6 (*d*); 36.9 (*t*); 27.7 (*t*); 27.0 (*t*); 20.2 (*q*); 18.8 (*t*); 18.5 (*q*); 18.1 (*q*); 16.4 (*q*). EI-MS: 236 (2, M^+), 193 (100), 191 (42), 175 (18), 121 (28), 107 (39), 93 (27). HR-ESI-MS: 237.2189 ($[M+H]^+$, $C_{16}H_{29}O^+$; calc. 237.2218).

Acetylation of Compound **4** *to 2-[(1*S*,2*R*,4a*R*,8a*R*)-1,2,3,4,4a,7,8,8a-Octahydro-1,2,4a,5-tetramethylnaphthalen-1-yl]ethyl acetate* (**5**). Compound **4** (49.6 mg) was dissolved in 1 ml of pyridine in a 20-ml scintillation vial, followed by the addition of 2 ml of Ac_2O. The mixture was stirred for 24 h at r.t. Reagents were removed by speed-vac and lyophilization. The residue was separated by silica gel CC on a *Biotage 25* + *M* column (25–63 μm, 60 Å, 25 × 150 mm) running at 25 ml/min using hexanes/AcOEt 95 : 5 over 1152 ml. 24-ml Fractions were collected and recombined based on TLC similarities providing 51.4 mg of pure **5**. ^{1}H-NMR ($CDCl_3$): 5.14 (br. *s*, 1 H); 4.06–3.94 (*m*, 2 H); 2.08–1.98 (*m*, 2 H); 1.98 (*s*, 3 H); 1.55 (*s*, 3 H); 0.96 (*s*, 3 H); 0.82 (*d*, *J* = 6.0, 3 H); 0.71 (*s*, 3 H). ^{13}C-NMR ($CDCl_3$): 171.2 (*s*); 144.3 (*s*); 120.7 (*d*); 61.1 (*t*); 47.6 (*d*); 39.0 (*s*); 38.5 (*s*); 37.5 (*d*); 36.9 (*t*); 36.4 (*t*); 27.7 (*t*); 26.9 (*t*); 21.2 (*q*); 20.2 (*q*); 18.8 (*t*); 18.1 (*q*); 18.1 (*q*); 16.3 (*q*). EI-MS: 278 (7), 219 (100), 217 (55), 190 (45), 175 (37), 95 (38). HR-ESI-MS: 579.4388 ($[2M+Na]^+$, $C_{36}H_{60}NaO_4^+$; calc. 579.4389), 301.2121 ($[M+Na]^+$, $C_{18}H_{30}NaO_2^+$; calc. 301.2144).

Jones *Oxidation of* **1** *to [(1*S*,2*R*,4a*R*,8a*R*)-1,2,3,4,4a,7,8,8a-Octahydro-1,2,4a,5-tetramethylnaphthalen-1-yl]acetic acid* (**6**). Using a 100-ml round-bottomed flask, *Jones* reagent (744 μl) was added to a mixture of **1** (200 mg) in acetone (100 ml) at 0°. The mixture was stirred, and the reaction was monitored by TLC for 1.5 h. The reaction was then quenched with 20 ml of deionized H_2O. The mixture was extracted twice with 20 ml of Et_2O (99.9% inhibitor free), and org. solvents were removed *in vacuo*. Reaction products were separated by normal-phase CC on a *Biotage 40* + *M* column (40–63 μm, 60 Å, 25 × 150 mm) running at 40 ml/min using a hexanes/Et_2O step gradient beginning with 100 : 0 to 90 : 10 over 1728 ml, 90 : 10 to 90 : 10 over 600 ml, 90 : 10 to 50 : 50 over 999 ml, and finishing with 100% Et_2O over 396 ml. 24-ml Fractions were collected and recombined based on TLC similarities into nine distinct compounds. The drying of *Fr. 8* provided 33.4 mg of pure **6**. ^{1}H-NMR ($CDCl_3$): 5.17 (br. *s*, 1 H); 2.42 (*d*, *J* = 14, 1 H); 2.33 (*d*, *J* = 14, 1 H); 1.55 (br. *s*, 3 H); 0.97 (*s*, 3 H); 0.87 (*d*, *J* = 7.0, 3 H); 0.76 (*s*, 3 H). ^{13}C-NMR ($CDCl_3$): 178.8 (*s*); 143.8 (*s*); 121.1 (*d*); 51.2, 48.1 (*d*); 43.2 (*t*); 41.3 (*s*); 38.6 (*s*); 37.6 (*d*); 36.5 (*t*); 27.5 (*t*); 27.0 (*t*); 20.0 (*q*); 19.4 (*t*); 18.2 (*q*); 17.6 (*q*); 16.4 (*q*). EI-MS: 250 (6, M^+), 235 (7), 191 (30), 190 (100), 175 (69), 121 (20). HR-ESI-MS (neg.): 249.1878 ($[M-H]^-$, $C_{16}H_{25}O_2^-$; calc. 249.1855).

CH_2N_2 Generation. An *Aldrich* (St. Louis, MO) *Mini Diazald* apparatus was used for the production of CH_2N_2 in Et_2O. Briefly, 2.5 g of KOH was dissolved in 4 ml of deionized water and placed in the reaction vessel, followed by the addition of 5 ml of EtOH. A separatory funnel containing 2.5 g of diazald dissolved in 22.5 ml of Et_2O was placed above the reaction vessel. The reaction vessel was warmed to 65° using a water bath, followed by the dropwise addition of the diazald soln. over a period of 50 min. The receiving flask and condenser cold finger were cooled using a dry-ice/acetone bath. The co-distilled CH_2N_2 in Et_2O soln. was stored in sealed vials at −20° until needed.

Esterfication of **6** *to Methyl [(1*S*,2*R*,4a*R*,8a*R*)-1,2,3,4,4a,7,8,8a-Octahydro-1,2,4a,5-tetramethylnaphthalen-1-yl]acetate* (**7**). Using a 100-ml round-bottomed flask, *Jones* reagent (744 μl) was added to a mixture of **1** (200 mg) in acetone (100 ml) at 0°. The mixture was stirred, and the reaction was monitored by TLC for 1.5 h. The reaction was then quenched with 20 ml of deionized H_2O. The mixture was extracted twice with 20 ml of Et_2O (99.9% inhibitor free), and the solvent was removed *in vacuo*. After removal of organics *in vacuo*, the residue was redissolved in 10 ml of Et_2O. To this soln. CH_2N_2 in Et_2O (*ca.* 8 ml) was added and left to react for 24 h. Compounds were separated with *Biotage 40* + *M* column (40–63 μm, 60 Å, 25 × 150 mm) running at 40 ml/min using hexanes: Et_2O step gradient beginning with 100 : 0 to 90 : 10 over 1728 ml and finishing with 90 : 10 to 90 : 10 over 400 ml. 24-ml Fractions were collected and recombined based on TLC similarities into two distinct fractions. Drying provided 115.9 mg of pure **7**. ^{1}H-NMR ($CDCl_3$): 5.13 (br. *s*, 1 H); 3.58 (*s*, 3 H); 2.38 (*d*, *J* = 14, 1 H); 2.26 (*d*, *J* = 14, 1 H); 1.52 (br. *s*, 3 H); 0.95 (*s*, 3 H); 0.85 (*d*, *J* = 6.8, 3 H); 0.73 (*s*, 3 H). ^{13}C-NMR ($CDCl_3$): 172.4; 143.9; 120.9; 51.2; 48.3; 43.1; 41.0; 38.5; 37.6; 36.5; 27.5; 27.0; 20.0; 19.4; 18.1; 17.4; 16.4. EI-MS: 264 (8, M^+), 221 (12), 191 (39), 190 (100), 121 (21). HR-ESI-MS (pos.): 287.1970 ($[M+Na]^+$, $C_{17}H_{28}NaO_2^+$; calc. 287.1987), 265.2166 ($[M+H]^+$, $C_{17}H_{29}O_2^+$; calc. 265.2168).

m*CPBA Oxidation of* **1** *to [(1a*S*,3a*R*,4*S*,5*R*,7a*R*,7b*R*)- and (1a*R*,3a*R*,4*S*,5*R*,7a*R*,7b*S*)-Decahydro-4,5,7a,7b-tetramethylnaphtho[1,2-*b*]oxiren-4-yl]acetaldehyde* (**8a** and **8b**, resp.). A soln. of 75.4 mg of **1** in 2 ml of CH_2Cl_2 was added to 83.3 mg of *m*CPBA in an ice bath for 1.0 h. The mixture was washed three times with 5 ml of 0.01M NaOH and once with 5 ml of deionized H_2O. Reaction products were separated by silica-gel CC on a *Biotage 40* + *M* column (40–63 μm, 60 Å, 25 × 150 mm) running at 40 ml/min using

hexanes/Et_2O step gradient beginning with 100:0 to 80:20 over 1200 ml, 80:20 to 50:50 over 1599 ml, and finishing with 50:50 to 100:0 over 600 ml. 24-ml Fractions were collected and recombined based on TLC similarities into three fractions with two distinct compounds. Drying provided 19.6 mg of pure **8a** and 11.9 mg of pure **8b**.

Data of Compound **8a**. ^{1}H-NMR ($CDCl_3$): 9.81 (*t*, *J* = 3.2, 1 H); 2.86 (*d*, *J* = 4.0, 1 H); 2.35–2.22 (*m*, 1 H); 1.18 (*s*, 3 H); 1.06 (*s*, 3 H); 0.92 (*d*, *J* = 6.8, 3 H); 0.78 (*s*, 3 H). ^{13}C-NMR ($CDCl_3$): 203.5 (*d*); 65.7 (*s*); 60.5 (*d*); 52.4 (*t*); 41.2 (*d*); 41.0 (*s*); 39.0 (*d*); 37.4 (*s*); 34.3 (*t*); 26.7 (*t*); 23.0 (*t*); 18.1 (*q*); 17.5 (*q*); 17.1 (*t*); 16.7 (*q*); 16.2 (*q*). EI-MS: 250 (0, M^+), 215 (24), 207 (100), 189 (48), 147 (17), 107 (19). HR-ESI-MS (pos.): 251.2031 ($[M+H]^+$, $C_{16}H_{27}O_2^+$; calc. 251.2011).

Data of Compound **8b**. ^{1}H-NMR ($CDCl_3$): 9.82 (*t*, *J* = 3.2, 1 H); 2.92 (*s*, 1 H); 2.43–2.32 (*m*, 2 H); 2.15–2.11 (*m*, 1 H); 1.17 (*s*, 3 H); 1.06 (*s*, 3 H); 0.94 (*d*, *J* = 6.4, 3 H); 0.75 (*s*, 3 H). ^{13}C-NMR ($CDCl_3$): 203.4 (*d*); 66.0 (*s*); 62.0 (*d*); 51.5 (*t*); 50.4 (*d*); 42.2 (*s*); 38.7 (*d*); 37.5 (*s*); 36.8 (*t*); 27.9 (*t*); 23.0 (*t*); 19.6 (*q*); 17.6 (*q*); 16.7 (*q*); 16.2 (*q*); 16.1 (*t*). EI-MS: 250 (1, M^+), 235 (60), 208 (18), 207 (100), 151 (31), 138 (44), 107 (27). HR-ESI-MS (pos.): 251.2019 ($[M+H]^+$, $C_{16}H_{27}O_2^+$; calc. 251.2011).

Hydroboration of **1** *to (4a*R,*5*S,*6*R,*8a*R*)-Decahydro-5-(2-hydroxyethyl)-1,5,6,8a-tetramethylnaphthalen-2-ol* (**9**). Compound **1** (209.5 mg) was dissolved in 5.8 ml of $BH_3 \cdot THF$ and transferred to a round-bottomed flask under magnetic stirring for 24 h. To this soln., 4 ml of 10% NaOH and 6 ml of H_2O_2 were added slowly in an ice bath for 6.0 h, as the mixture warmed to 20°. This mixture was extracted three times with 10 ml of AcOEt. The combined org. extracts were washed once with 25 ml of brine and dried ($MgSO_4$). After decanting and removal of organics *in vacuo,* the (332.3 mg) of residue was separated by normal-phase CC on a *Biotage 40+M* column (40–63 μm, 60 Å, 40 × 150 mm) running Et_2O at 40 ml/min over 1152 ml, followed by an AcOEt wash over 300 ml. 24-ml Fractions were collected and recombined based on TLC similarities into three distinct fractions (*A–C*). *Fr. C* provided 25.6 mg of pure **9**. ^{1}H-NMR (CD_3OD): 3.57–3.40 (*m*, 2 H); 3.28–3.18 (*m*, 2 H); 2.04–1.95 (*m*, 1 H); 0.83 (*d*, *J* = 6.8, 3 H); 0.81 (*d*, *J* = 6.8, 3 H); 0.75 (*s*, 3 H); 0.70 (*s*, 3 H). ^{13}C-NMR ($CDCl_3$): 72.6 (*d*); 58.6 (*t*); 54.4 (*d*); 51.8 (*d*); 41.7 (*t*); 40.7 (*t*); 40.0 (*d*); 39.1 (*s*); 38.7 (*s*); 37.6 (*t*); 28.4 (*t*); 21.9 (*t*); 18.7 (*q*); 16.6 (*q*); 14.8 (*q*); 10.6 (*q*). HR-ESI-MS (pos.): 277.2129 ($[M+Na]^+$, $C_{16}H_{30}NaO_2^+$; calc. 277.2144).

Aedes aegypti *Mosquito Bioassay Method Used in Biting-Deterrency Studies. A. aegypti* (L.) (red-eye Liverpool strain) used in the study were from colonies maintained at the *Walter Reed Army Institute of Research*, Silver Spring, MD. The insects were reared [11] by feeding larvae ground tropical fish flakes (*Tetramin Tropical Fish Flakes*, *Tetra Sales*, Blacksburg, VA, www.tetra-fish.com). Adults were maintained in a photoperiod of 12:12 (light/day) h at 27° and 80% rel. humidity (RH) with cotton pads moistened with 10% aq. sucrose soln. Mated females were 5–15 days old when they were used in bioassays. *Ae. aegypti* females had access only to water 24 h, and neither food nor water for another 24 h before testing. All tests were conducted 4–6 h after the beginning of photophase (1000 h).

All experiments were conducted by using a six-celled *in vitro Klun & Debboun* (*K & D*) module bioassay system developed by *Klun et al.* [12] for quant. evaluation of bite-deterrent properties of candidate compounds for human use. *Dethier et al.* [13] defined a repellent as a chemical which causes insects to make oriented movement away from its source, and a deterrent was defined as a chemical which inhibits feeding or oviposition when present in a place where insects would, in its absence, feed or oviposit. The bioassay method we used in this research specifically measured biting (feeding) deterrent properties of chemicals. Therefore, the compounds identified here are best defined as being deterrents and not repellents; although, in another bioassay mode, they might also exhibit a repellent effect. Full experimental details have been described in [14][15]

Aedes aegypti *Mosquito and Adult Bioassay Method Used in Toxicity Studies.* The Orlando strain of *Ae. aegypti* was reared in the insectary of the *Mosquito and Fly Research Unit* at *Center for Medical, Agricultural, and Veterinary Entomology*, USDA-ARS and was used for adult toxicity experiments. Eggs were hatched by placing a square of a paper towel with eggs in a flask filled with 1000 ml of distilled water containing 40 mg of larval diet (3:2 brewer's yeast/liver powder (*MP Biomedicals*, Irvine, CA)). The hatched larvae were held overnight in the flask, and 200 larvae were transferred to a 4-l plastic tray containing 2 l of distilled water. Larval diet was added to each tray according to the following schedule: day 1, 80 mg; day 3, 40 mg; day 4, 80 mg; day 5, 120 mg; and day 6, 150 mg. Mosquitoes were reared in an environmental chamber set with a temp. profile representing a simulated summer day regime (ranging

from 22 to 30°) and 80% RH. Incandescent lighting was set to a crepuscular profile with a photoperiod of 14 : 10 (light/day) h, including 2 h of simulated dawn and 2 h of simulated dusk. Adults were held in a screened cage and provided 10% sucrose *ad libitum*. Bovine blood in 1% heparin that had been placed in a pig intestine and warmed to 37° was provided to adults twice a week. Eggs were collected on paper towels (*Vasco Brands*, Elmira, NY) that lined the rim of water containers. These egg-laden papers were air dried at 27° and 80% RH for 24 h and stored in containers with 100% humidity for 3–30 days. When needed, eggs were hatched under vacuum to ensure hatching in a narrow window of time and larvae were reared in containers as described above. To determine precisely the toxicity of each chemical against female *Ae. aegypti*, each chemical was serially diluted in acetone and topically applied to individual mosquitoes. Prior to insecticide application, 5–7-day-old females were briefly anaesthetized for 30 s with CO_2 and placed on a 4° chill table (*BioQuip Products*, Rancho Dominguez, CA). A droplet of 0.5 µl of insecticide soln. was applied to the dorsal thorax using a 700 series syringe and a *PB 600* repeating dispenser (*Hamilton*, Reno, NV). Six concentrations providing a range of 0–100% of mortality were used on 25–30 females per concentration. Tests were replicated three times. Control treatments with 0.5 µl of acetone alone gave control mortality rates of $<10\%$. After treatment, mosquitoes were kept in plastic cups and supplied with 10% sucrose soln. for 24 h before mortality was recorded. Temp. and humidity were maintained at 26° and 80% RH, resp. At least five concentrations were used for each bioassay. Every bioassay was conducted at 27° and 80% RH and replicated three times. Bioassay data were analyzed, and LD_{50}/LD_{95} values were calculated using PoloPlus probit and logit analysis software (*LeOra Software*, Petaluma, CA). LD_{50} and LD_{95} values were judged as significantly different ($P \leq 0.05$), if the confidence intervals did not overlap.

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